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“One-electron oxidation and reduction of glycosaminoglycan chloramides: a kinetic study”

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Abstract

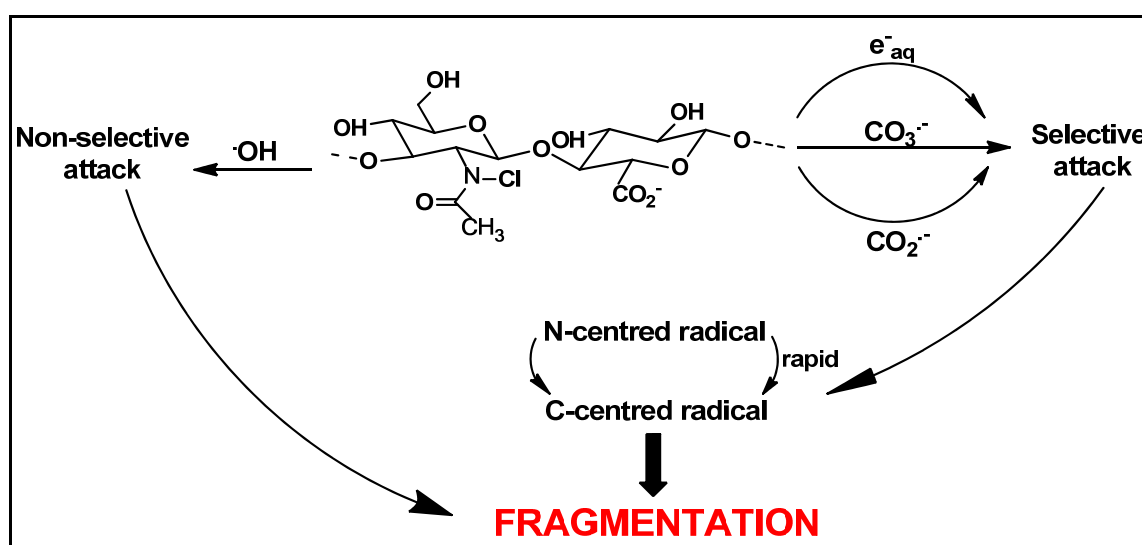
Hypochlorous acid and its acid-base counterpart, hypochlorite ions, produced in inflammatory conditions, may produce chloramides of glycosaminoglycans, the latter being significant components of the extracellular matrix (ECM). This may occur through the binding of myeloperoxidase directly to the glycosaminoglycans. The N-Cl group in the chloramides is a potential selective target for both reducing and oxidising radicals, leading possibly to more efficient and damaging fragmentation of these biopolymers relative to the parent glycosaminoglycans. In this study, the fast reaction techniques of pulse radiolysis and nanosecond laser flash photolysis have been used to generate both oxidizing and reducing radicals to react with the chloramides of hyaluronan (HACl) and heparin (HepCl).

The strong reducing formate radicals and hydrated electrons were found to react rapidly with both HACl and HepCl with rate constants of $(1-1.7) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $(0.7-1.2) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for formate radicals and $2.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ and $7.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for hydrated electrons, respectively. The spectral characteristics of the products of these reactions were identical and were consistent with initial attack at the N-Cl groups, followed by elimination of chloride ions to produce nitrogen-centred radicals, which re-arrange subsequently and rapidly to produce C-2 radicals on the glucosamine moiety supporting an earlier EPR study by Rees et al. *J. Am. Chem. Soc.* **125**: 13719-13733; 2003.

The oxidising hydroxyl radicals also reacted rapidly with HACl and HepCl with rate constants of $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $1.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, with no evidence from this data for any degree of selective attack for the N-Cl group relative to the N-H groups and other sites of attack. The carbonate anion radicals were found much slower with HACl and HepCl than hydroxyl radicals ($1.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $8.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively) but significantly faster than with the parent molecules ($3.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $5.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, respectively). These findings suggest that these potential *in vivo* radicals may react in a site-specific manner with the N-Cl group in the glycosaminoglycan chloramides of the ECM, possibly to produce more efficient fragmentation.

This is the first study therefore to conclusively demonstrate that reducing radicals react rapidly with glycosaminoglycan chloramides in a site-specific attack at the N-Cl groups, probably to produce a 100 % efficient biopolymer fragmentation process. Although less reactive, carbonate radicals, which may be produced *in vivo* via reactions of peroxynitrite with serum levels of carbon dioxide, also appear to react in a highly site-specific manner at the N-Cl group. It is not yet known if such site-specific attacks by this important *in vivo* species lead to a more efficient fragmentation of the biopolymers than would be expected for attack by the stronger oxidising species, the hydroxyl radical. It is clear however that the N-Cl group formed in inflammatory conditions in the extracellular matrix does present a more likely target for both reactive oxygen species and for reducing species than the N-H groups in the parent glycosaminoglycans.

Graphical abstract



Introduction

The extracellular matrix (ECM) is made up of huge multi-molecular complexes with arrays of link proteins and aggrecan molecules along a central hyaluronan backbone. Hyaluronan (HA) is bound by a number of ECM and cell surface proteins and is a particularly important component of the ECM [1-5]. HA is involved in moderating many cellular processes, including proliferation, migration, adhesion and apoptosis [6-11]. Large molecular mass fragments of HA are involved in space-filling and immunosuppressive roles, whilst smaller HA fragments have been shown to be pro-inflammatory and angiogenic; oligosaccharides may be involved in cell signalling (reviewed in [12]).

Oxidative damage of the extracellular components by either enzymatic or non-enzymatic pathways may have implications for the initiation and progression of a range of human diseases. These include arthritis, kidney disease, cardiovascular disease, lung disease, periodontal disease and chronic inflammation. Oxidative damage to HA by reactive oxidative species (ROS) has been the subject of numerous studies (reviewed in [13]). The potential mechanism of oxidative damage to the ECM and its role in human pathologies has also been discussed in a recent review [14].

Our previous studies on HA have measured HA fragmentation yields as a proportion of quantifiable fluxes of free radicals produced by ionising radiation. In this way, the efficiencies of fragmentation of HA by a range of free radicals and ROS, including hydroxyl radicals, carbonate radicals, dibromide and dichloride radical anions and peroxynitrite were determined [15,16].

The fragmentation of HA and other glycosaminoglycans has also been investigated intensively by Davies and co-workers using both electron paramagnetic resonance (EPR) spectroscopy and sensitive PAGE techniques to show peroxynitrous acid, carbonate and hydroxyl radicals react largely in a site-specific process to produce an array of HA fragments, in a “ladder-type display” each separated from the neighbour by the molecular mass of the repeating disaccharide unit in HA [17,18]. Similar site-selective fragmentation was also observed when glycosaminoglycan chloramides (formed through reaction with hypochlorite) were reduced by copper (I) ions and superoxide anion radicals [19,20].

The formation of chloramides and chloramines from the reaction of hypochlorite with amides and amines respectively was demonstrated in an early study [21] and is suggested to be a key process in inflammation, in which hypochlorite (from myeloperoxidase) may produce glycosaminoglycan chloramides. *In vitro* studies of the reactions of hypochlorite with glycosaminoglycans have indeed demonstrated that chloramides are produced in yields and rates of reaction which are dependent upon both pH and the ratio of hypochlorite to glycosaminoglycan concentrations [22, 23] and have also shown that such derivatives may accelerate the fragmentation of glycosaminoglycans within the ECM [22]. Chloramides are weak oxidising agents and are therefore, potential biological targets for reducing radicals and other reducing agents. Indeed, it has been shown that superoxide radicals cause the fragmentation of HA via a metal ion- catalysed reaction with its chloramide derivative [19].

There is however, only a limited amount of kinetic data available on the direct measurement of the rate constants for the reactions between free radicals and glycosaminoglycans and none for the reactions with their chloramides. The technique of pulse radiolysis has been used previously to measure rate constants for the reactions of the hydroxyl radical ($\cdot\text{OH}$), the hydrated electron, (e^-_{aq}) and the hydrogen atom ($\text{H}\cdot$) with HA [24]. In a more recent study, this was extended to reactions of the dichloride radical anion ($\text{Cl}_2^{\cdot-}$), the dibromide radical anion ($\text{Br}_2^{\cdot-}$), the carbonate radical ($\text{CO}_3^{\cdot-}$) and the azide radical ($\text{N}_3^{\cdot-}$) [16].

Strong reducing agents such as the hydrated electron, e^-_{aq} , may be expected to react rapidly with chloramines and chloramides. Pulse radiolysis studies of the simplest chloramine, NH_2Cl , showed that it reacts with the hydrated electron at diffusion-controlled rates ($k = 2.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [25,26]. In more recent pulse radiolysis studies of the chloramines and chloramides of amino acid derivatives [27] and of chloro- and bromo- derivatives of model compounds, such as N-bromoglutarimide (NBG) and N-bromosuccinimide (NBS) [28], hydrated electrons were also shown to react at near diffusion-controlled rates. In the latter study, superoxide radicals were also reacted with NBG and NBS and found to follow complex chain reaction pathways. In the case of N-chlorosuccinimide, a direct kinetic measurement could be made, yielding a rate constant of $8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. An indirect method, based on EPR data, has also allowed an estimate of the rate

constant for the reaction of superoxide radicals with taurine monochloramine to be made ($k = 5\text{-}6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) [29].

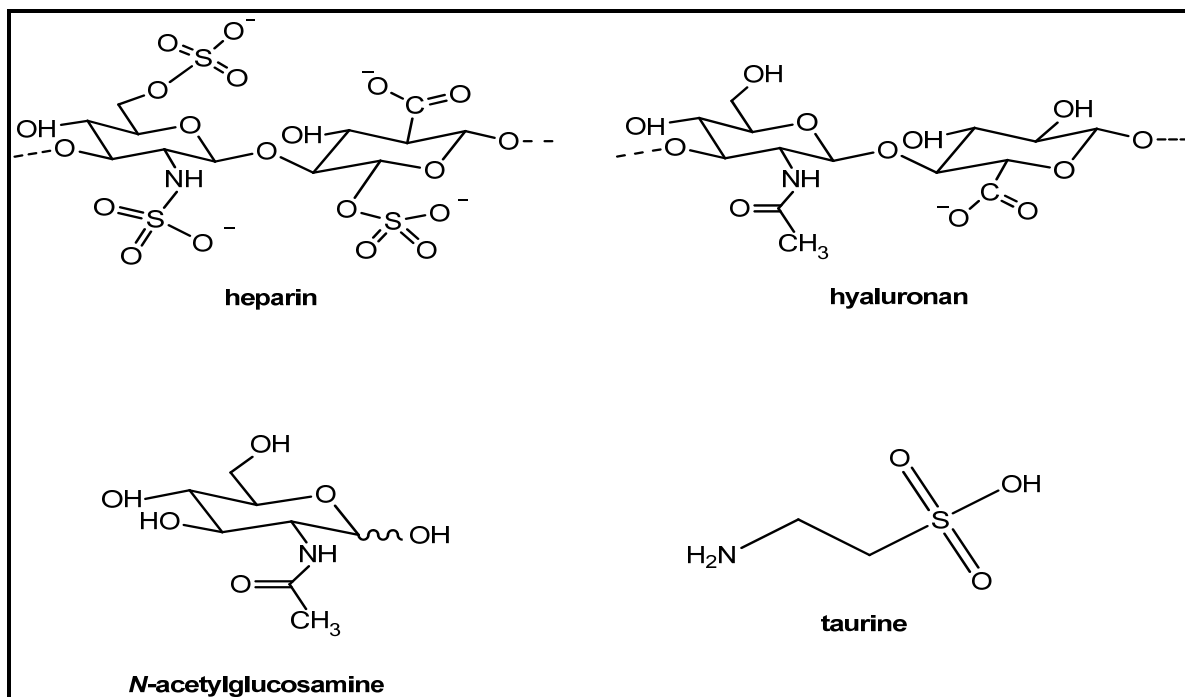
Consequently, in this study the fast reaction techniques of pulse radiolysis and laser flash photolysis have been used to directly measure the rate constants for the reactions of both oxidising and reducing radicals with the chloramides of HA and heparin, the latter being the most heavily sulphated glycosaminoglycan, which provides an opportunity to investigate both the effect of charge and sulphate group on the reactivity with free radicals. The biologically-relevant hydroxyl and carbonate radicals have been selected as oxidising species in this study whilst the strong reducing agents, hydrated electron and formate radicals have been selected as models for the *in vivo* but less strongly reducing agents such as superoxide and the disulphide radical anions of glutathione. The kinetic data has also allowed the degree of selectivity to be determined, through reaction at the N-Cl group, a potential route to efficient fragmentation of the biopolymers in inflammatory conditions in the ECM.

Materials and methods

a) Materials

Sodium formate, hypochlorous acid, *tert*-butanol and sodium bicarbonate were all of the highest quality available (Sigma Aldrich). HA (both 80 kDa and 0.8 MDa) were gifts from Novozymes and the heparin sodium salt (Alfa Aesar) was of research grade. N-acetylglucosamine and taurine were purchased from Sigma

Aldrich (see Scheme 1 for the structures of HA, heparin , N-acetylglucosamine and taurine). Water for buffer preparation was prepared by an Elga system (resistivity 18 M Ω).



Scheme 1

b) Preparation of chloramines and chloramides

The chloramides were prepared from each of HA (4 mg/ml), heparin (6.4 mg/ml), taurine (1.25 mg/ml) and glucosamine (1 mg/ml) by reacting with pH- adjusted HOCl (2-9 mM) at 37°C and were terminated by addition of excess taurine to remove unreacted HOCl. Polymer chloramide reactions were carried out for a duration of 5 hrs at 37°C, which was followed by dialysis in 0.1 M phosphate buffer to remove taurine and taurine chloramines. The preparations resulted in a mixture containing both the chloramide and the parent glycosaminoglycan. Typical preparations comprised 60 % HA and 40 % HA chloramide and, for

heparin preparations, 80 % heparin chloramide, 20 % heparin. Taurine monochloramine (TauCl) solutions were prepared by the addition of equal concentrations of taurine and hypochlorite at pH 7.4. The reaction was complete within the mixing time. N-Acetylglucosamine chloramide was prepared in a reaction mixture containing 1 mM hypochlorite and 10 mM N-acetylglucosamine for 90 minutes at 37°C to produce 1 mM of the chloramide. The synthesised chloramides and taurine monochloramine were used in pulse radiolysis and laser flash photolysis as soon as possible thereafter. The chloramide concentrations, prior to pulse radiolysis, were determined using the TNB assay [30]. Solutions for the laser flash photolysis and pulse radiolysis experiments were made up in 0.01 M phosphate buffer and were saturated as appropriate with research grade nitrous oxide, argon or nitrogen , as appropriate. In the case of the carbonate radical experiments, air saturated solutions were used.

c) Pulse radiolysis and laser flash photolysis

Pulse radiolysis studies were carried out using both the linear accelerator at the Curie Institute, Orsay, France and the picosecond laser triggered electron accelerator ELYSE at Laboratoire de Chimie Physique, Université Paris Sud . In the Curie set-up, free radicals were generated by 600-1200 ns pulses of 4 MeV electrons. Radiation doses generated per pulse were calibrated from the absorbance of the thiocyanate radical $(\text{SCN})_2^{\cdot -}$ produced from the pulse radiolysis of 10 mM air saturated thiocyanate ion solutions in 10 mM phosphate buffer at pH 7.0 where $G(\text{SCN})_2^{\cdot -} = 0.30 \mu\text{mol} \cdot \text{J}^{-1}$, $\epsilon = 7400 \text{ M}^{-1}\text{cm}^{-1}$ at 472 nm.

The ELYSE laser-triggered accelerator delivered 15 ps electron pulses with energy 4-9 MeV at 3 nC per pulse with a repetition rate of 1 to 50 Hz. The doses generated by the laser-triggered accelerator were in the range 19-30 Gy.

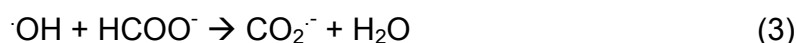
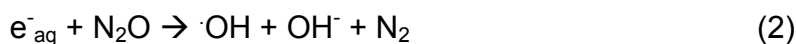
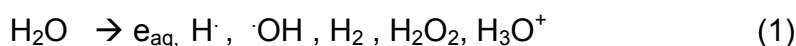
For laser photoexcitation experiments, 1 ml samples were excited using the 4th harmonic (266 nm) of a Q-switched Nd-YAG laser (Brilliant B, Quantel) in a 1 ml quartz cuvette of 1 cm pathlength. The energy output of each laser pulse was approximately 40 mJ and pulses were 6-8 ns in duration. Data was collected using an Applied Photophysics LKS-60 flash photolysis instrument with detection system at right angles to the incident laser beam. The probe light (150 W xenon lamp) was passed through a monochromator before and after passage through the sample. Absorbance changes were measured using a photomultiplier tube and kinetic transients were typically collected over 200 ms. For measurements over faster timescales (typically < 1 ms), the output of the xenon arc lamp was pulsed using a xenon arc pulser (Applied Photophysics) and transients were measured using an Infiniium oscilloscope model no. 54830B (Agilent Technologies).

Results and Discussion

Reduction by formate radicals

Formate radicals were produced by both pulse radiolysis and by laser flash photolysis. In the former technique, nitrous oxide saturated solutions of 0.1 M formate containing a range of concentrations of HCl (2.0×10^{-4} M to 9×10^{-4} M),

HepCl (1.2×10^{-3} M to 4×10^{-3} M), TauCl (8.0×10^{-5} M to 1.6×10^{-4} M) and N-acetylglucosamine chloramide (GlcAcNCl) (1.1×10^{-4} M to 1.1×10^{-3} M) were pulse-irradiated. Under these conditions, all primary radicals produce formate radicals in a yield of $0.62 \mu\text{m J}^{-1}$, as follows:



Laser flash photolysis was also used to produce the formate radical in yields of up to 4.1×10^{-5} M. This was achieved by excitation at 266 nm of nitrogen saturated solutions containing 30 mM persulphate ($\text{S}_2\text{O}_8^{2-}$) and 10-50 mM formate (HCOO^-). Upon excitation at 266 nm, persulphate (extinction coefficient of approximately $20 \text{ M}^{-1} \text{ cm}^{-1}$) produces sulphate anion radicals ($\text{SO}_4^{\cdot-}$) with a quantum efficiency close to 2 [31-33] (reaction 5). $\text{SO}_4^{\cdot-}$ has a peak of maximum absorbance at 455 nm but several different values have been measured for its extinction coefficient [31-40]. The reasons for the wide range of reported values have been discussed in detail [33, 36] and based on these findings, a value of $1600 \text{ M}^{-1} \text{ cm}^{-1}$ at 455 nm has been used in the current work.



In the presence of formate, $\text{SO}_4^{\cdot-}$ oxidises formate to $\text{CO}_2^{\cdot-}$, with a rate constant for reaction (6) of $(1.1 - 1.7) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [41, 42].



At 10 mM formate, the decay of the $\text{SO}_4^{\cdot-}$, monitored at 455 nm, was complete within 2-3 μs (Figure 1a), consistent with the above second-order rate constants. Figure 1b shows the production of formate observed at 270 nm, on the same timescale. Although the presence of very low concentrations of oxygen, up to 1 μM can not be discounted as an impurity in the nitrogen used to de-aerate the solution, this concentration would only react with a maximum of 2-4% of the formate radical concentration produced (see below) to form superoxide radicals. The latter would be much longer-lived than formate radicals – however there was no indication of such a long-life component. It seems likely therefore that the formation of either superoxide radicals or peroxy radicals to any significant extent can be discounted in these experiments.

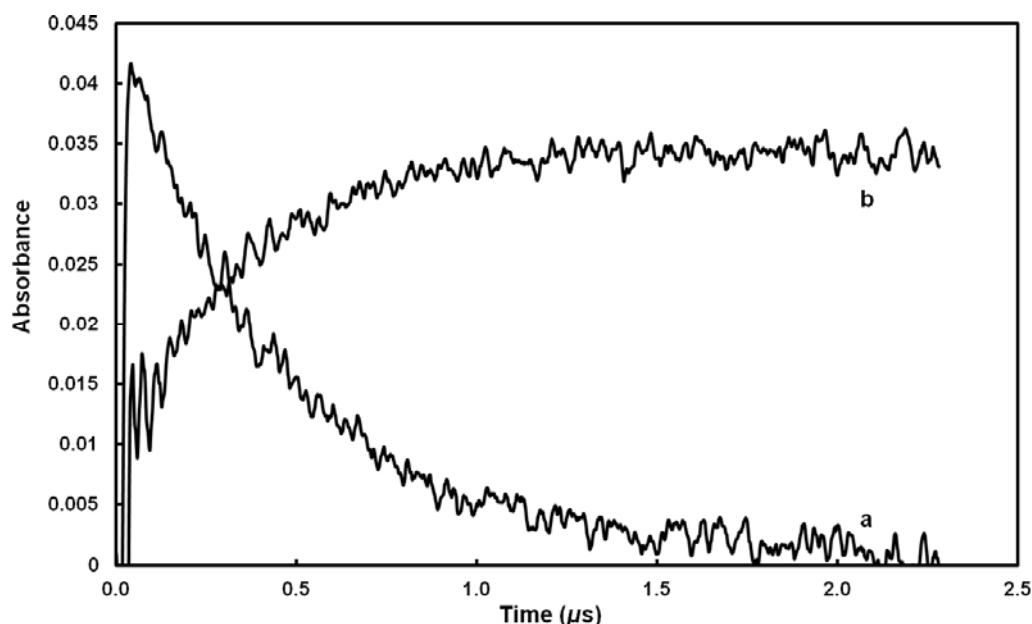


Figure 1 Transient absorbance changes on laser flash photolysis of a nitrogen saturated 30 mM persulphate, 10 mM formate solution at pH 7.4: a) 450nm ; b) 270nm.

Addition of HA, heparin, HA chloramide and heparin chloramide at concentrations of up to 4 mM did not increase the rate of decay of $\text{SO}_4^{\cdot-}$, indicating an upper limit of $4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for reactions with these substrates. Hence, it is unlikely that reactions of the sulphate radical with these glycosaminoglycans and their chloramide derivatives will compete significantly with the formation of $\text{CO}_2^{\cdot-}$ at the formate concentrations employed. Using an extinction coefficient of $1600 \text{ M}^{-1} \text{ cm}^{-1}$ at 455 nm for the sulphate radical, typical initial yields at the end of the laser pulse were in the range $(2.5 - 4.1) \times 10^{-5} \text{ M}$, producing identical yields of the formate radical. In the presence of HA and heparin chloramides, over a timescale up to 50 μs , an increase in absorbance could be observed in the 300-400 nm range, as shown in Figures 2a and 2b at

320 nm. There is a rapid decrease in absorbance over 2-3 μs corresponding to the conversion of sulphate anion radicals to formate radicals, followed by a slower increase in absorbance, attributable to the product of the reaction of formate radicals with the chloramides (reaction (7)):

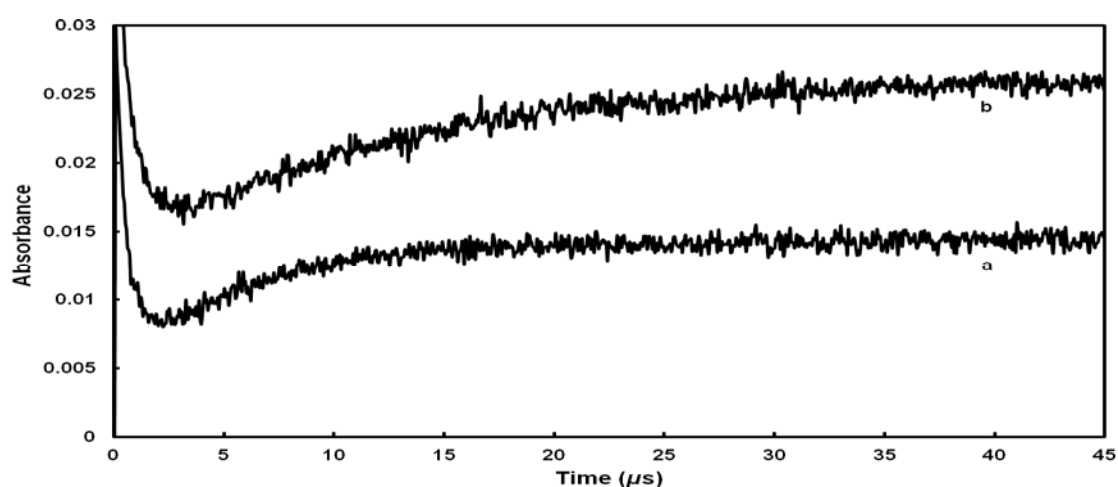


Figure 2 Transient absorbance changes at 320nm on laser flash photolysis of a nitrogen saturated solution of 30 mM persulphate, 10 mM formate solution containing ; a) 1 mM HACl ; b) 1mM HepCl.

Although all the chloramide solutions contain unsubstituted glycosaminoglycan, the latter are unlikely to react with reducing radicals, as demonstrated in an earlier pulse radiolysis study of hyaluronan. Figure 3 shows the spectra in the 300-400 nm region measured at the end of the latter reaction for both HACl and HepCl solutions, which show maxima 320 / 360nm for HepCl and a much less characteristic spectrum for HACl .

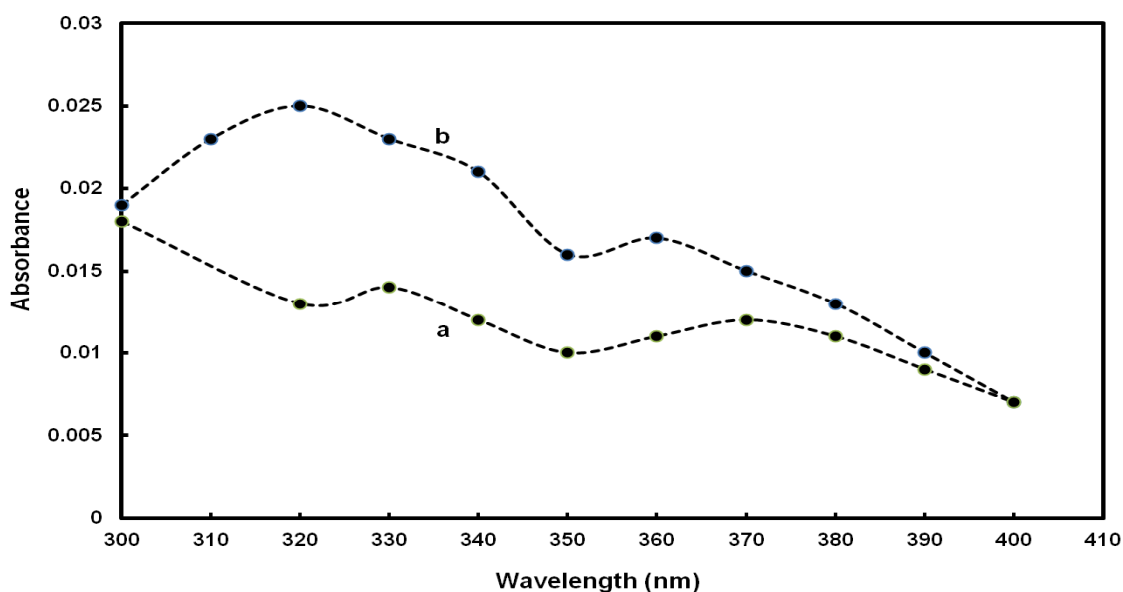


Figure 3 Spectra at 50 μ s after laser flash photolysis of a nitrogen saturated solution of 30 mM persulphate, 10 mM formate solution containing ; a) 1 mM HACl ; b) 1mM HepCl.

The second-order rate constants for reaction (7) were obtained from $\log (A_{\infty} - A_t)$ versus time plots (from kinetic traces, such as those in Figure 2) consistent with pseudo first-order kinetics, where A_{∞} and A_t are absorbances at the end of the reaction and times t . The resulting values were $(1.7 \pm 2.0) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $(1.2 \pm 1 \times 10^8) \text{ M}^{-1} \text{ s}^{-1}$ for HACl and HepCl respectively.

From Figure 3, the apparent extinction coefficients could be calculated based on a formate radical yield of $2.5 \times 10^{-5} \text{ M}$. These values were 480 (370 nm) and 680 (360 nm) $\text{M}^{-1} \text{ cm}^{-1}$ for HACl and HepCl respectively. However, the true values are likely to be significantly higher due to competing reactions, particularly the self-reactions of formate and product radicals. Using the IBM Chemical Kinetics Simulator v1.01 programme and assuming rate constants for the self-reaction of formate and product radicals to be $2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (the latter measured in a

separate experiment but not shown) and $5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (assumed for the self-reaction of product radicals) respectively, more realistic extinction coefficients of 930 (370 nm) and 1130 (360 nm) $\text{M}^{-1} \text{ cm}^{-1}$ for HAcI and HepCl respectively were estimated.

In pulse radiolysis experiments, the competing self-reactions of formate and product radicals were also significant. In order to gain accurate measurements of rate constants and also extinction coefficients at key wavelengths of the products, experiments were repeated at higher concentrations of chloramide. Figure 4 shows kinetic traces at 330 nm for HepCl concentrations in the range $(1.15 - 4.6) \times 10^{-3} \text{ M}$. These show a clear concentration dependence of the rates of reaction. Plots of $\log (A_{\infty} - A_t)$ versus time yielded a linear $k (\text{s}^{-1}) \propto [\text{HepCl}]$ plot as shown in the inset to Figure 4. From the slope, a second order rate constant of $(6.8 \pm 1.0) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ was calculated, a value similar to that of $(1.2 \pm 0.5) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ found in the laser flash photolysis experiments. Figure 4 also shows the effect of the competing self-reactions more clearly. At the concentrations of formate radicals produced in these experiments ($2.0 \times 10^{-5} \text{ M}$), the apparent extinction coefficient at 330 nm at 50 μs after the pulse can be calculated as 1700-1800 $\text{M}^{-1} \text{ cm}^{-1}$, similar to the value of 1700 $\text{M}^{-1} \text{ cm}^{-1}$ found at the same wavelength in the laser experiments. For the highest HepCl concentration of $4.6 \times 10^{-3} \text{ M}$, where competing reactions are minimised, the formate radical product is produced in a much shorter time of about 6 μs with an extinction coefficient of 2300 $\text{M}^{-1} \text{ cm}^{-1}$.

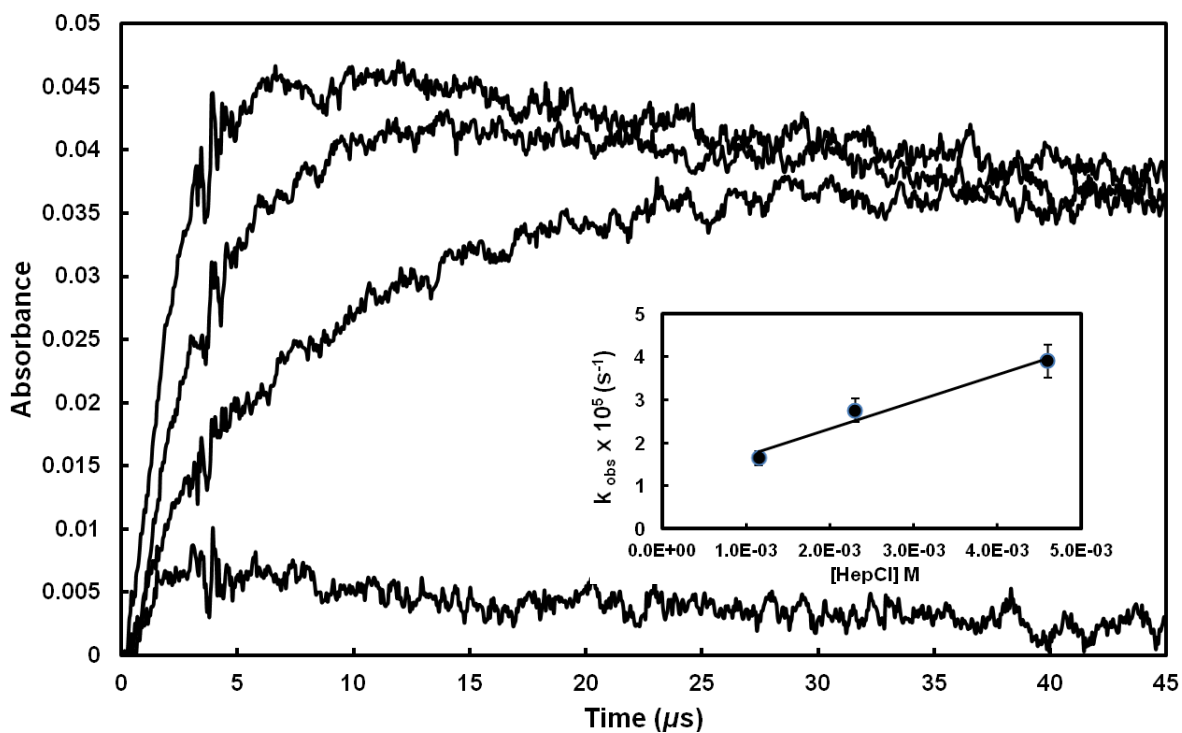


Figure 4 Transient absorbance changes at 330 nm on pulse radiolysis of nitrous oxide saturated solutions of 0.1 M formate and HepCl ($1.15 \times 10^{-3} \text{ M}$ to $4.6 \times 10^{-3} \text{ M}$), $[\text{CO}_2^-] = 2.0 \times 10^{-5} \text{ M}$). The inset shows a plot of $k \text{ (s}^{-1}\text{)}$ v $[\text{HepCl}]$.

Experiments with HAcI were limited to significantly lower concentrations than for HepCl and produced data which supported the kinetic and spectral data already determined in the laser flash photolysis experiments described above.

Limited pulse radiolysis experiments were carried out on the chloramide of the model monosaccharide, N-acetylglucosamine (N-AcGlcCl). Figure 5 shows kinetic traces, monitored at 330 nm, at two concentrations ($1.1 \times 10^{-4} \text{ M}$ and $1.1 \times 10^{-3} \text{ M}$) of the sugar. The rate of formation of the radical product is significantly faster at the higher N-AcGlcCl concentration than at the lower concentration

although a difference of 10 is not so apparent. At the higher concentration, some distortion at early times from the pulse is observed whilst at the lower concentration, the rate of growth is moderated by a significant self-reaction of the radicals. The rates of reaction are however dependent upon [N-AcGlcCl] and clearly faster, as might be expected for a smaller molecule, than those of the polymer chloramides, with a second-order rate constant of $(1.8 \pm 0.4) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ being estimated. At the highest [N-AcGlcCl], the extinction coefficient at 330 nm for the product was calculated as $2500 \text{ M}^{-1} \text{ cm}^{-1}$, consistent with value of $2300 \text{ M}^{-1} \text{ cm}^{-1}$ found above in pulse radiolysis of high concentrations of HepCl.

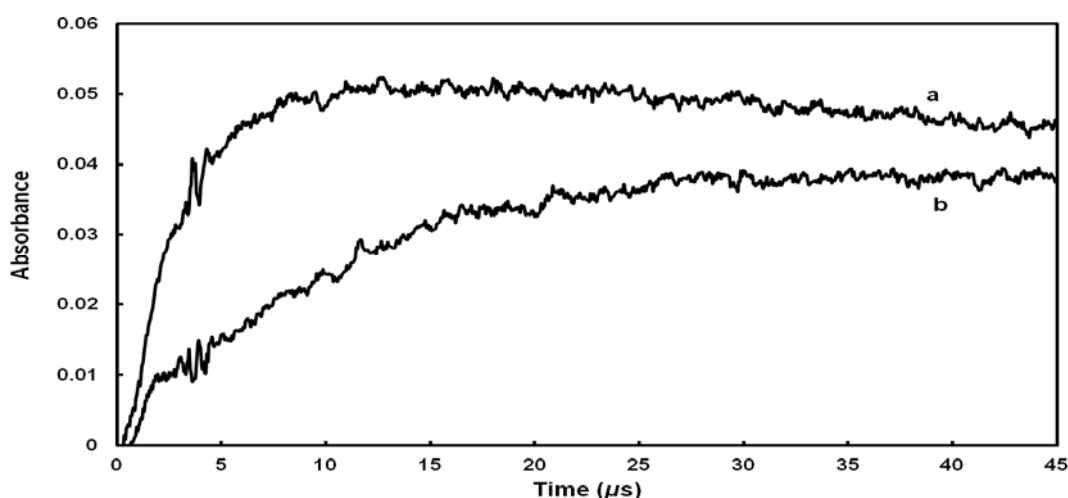


Figure 5 Transient absorbance changes at 330 nm on pulse radiolysis of nitrous oxide saturated solutions of 0.1 M formate and N-AcGlcCl ($1.1 \times 10^{-4} \text{ M}$ to $1.1 \times 10^{-3} \text{ M}$), $[\text{CO}_2^{\cdot-}] = 2.0 \times 10^{-5} \text{ M}$).

Reduction by the hydrated electron

Hydrated electrons, e_{aq}^- , were generated by pulse radiolysis of argon-saturated 0.1 M *tert*-butanol solutions containing a range of chloramide concentrations. Radiolysis of aqueous solutions produces the primary radicals, e_{aq}^- , hydrogen atoms ($H\cdot$) and hydroxyl radicals, ($\cdot OH$), as shown in reaction (1). In the presence of *tert*-butanol, hydroxyl radicals are scavenged to produce non-reactive species, allowing the reactivity of the hydrated electron to be studied independently. The rates of reaction of e_{aq}^- with taurine monochloramine, (TauCl), HA chloramide (HACl) and heparin chloramide (HepCl) were determined from the decay of absorbance at 660 nm, attributable to the hydrated electron. The decays were followed typically for up to 2 μs after the pulse and were found to obey first-order kinetics. Figure 6 shows the kinetic trace for the reaction of hydrated electrons with HACl at concentrations in the range, 1×10^{-4} M to 1×10^{-3} M. The rate of decay increases with increases in [HACl] as shown in the inset to Figure 6, from which a second-order rate constant of $(2.2 \pm 0.3) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ can be determined. Similar experiments were carried out with HepCl and taurine monochloramine (TauCl). All the hydrated electron rate constants are summarised in Table 1. As shown already for the reaction of formate radicals with HACl and HepCl, transient spectra in the wavelength range, 300 nm-400 nm were also observed at the end of the hydrated electron reactions. Although these spectra were of poorer quality than those obtained in the laser flash photolysis experiments, the free radical products are formed much faster and (allowing for the intrinsic decay of the hydrated electron), in about 70% yield. They did not

therefore require kinetic simulations to allow for decay of the free radical products to estimate extinction coefficients. The spectra (not shown) showed broad absorption in the range 300 -350 nm tailing off at wavelengths greater than 350nm, with an estimated extinction coefficient of $1700 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$ at 320 nm. The laser and pulse radiolysis data taken together provide support for the formation of C-centred radicals and not N-centred radicals as discussed below.

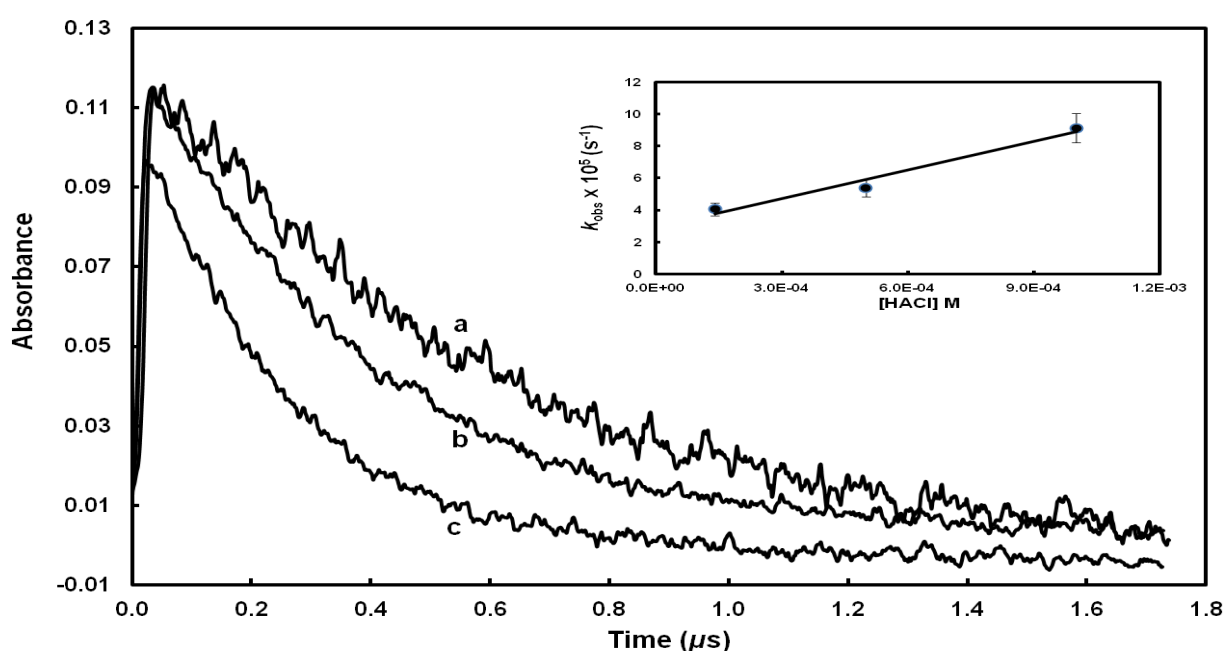


Figure 6 Decay of absorbance at 660 nm on pulse radiolysis of an argon saturated solution of 0.1 M t-butanol containing ; a) $1.0 \times 10^{-4} \text{ M}$, b) $5.0 \times 10^{-4} \text{ M}$ and c) $1.0 \times 10^{-3} \text{ M}$ HAcI at pH 7.4. The inset is a plot of $k \text{ (s}^{-1}\text{)}$ values v $[\text{HAcI}]$.

"Substrate, S"	$k (e_{\text{aq}} + \text{S}) \text{ M}^{-1} \text{ s}^{-1}$
HAcI	2.2×10^9
HepCl	7.2×10^8
TauCl	9.2×10^9

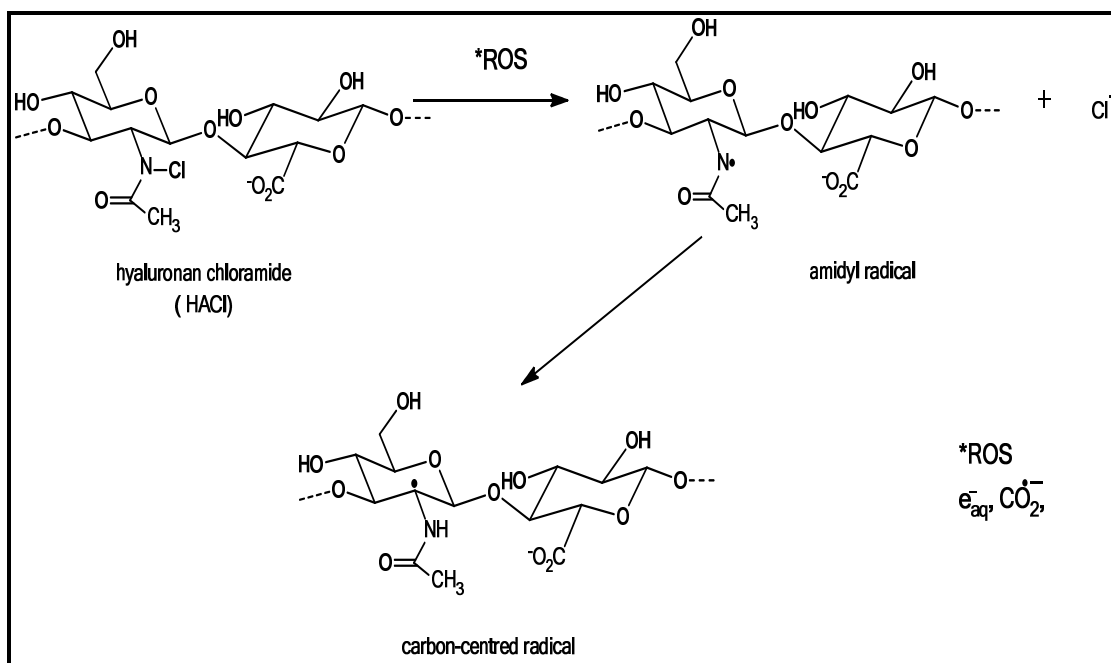
Table 1 Rates of reaction of the hydrated electron with HAcI, HepCl and TauCl.

From the above experiments with the reducing radicals of formate and hydrated electron, it is clear that both species react rapidly with the chloramides. As expected from the respective standard reduction potentials of the hydrated electron (-2.9 V, [43]) and carbon dioxide (-1.9 V, [44]), the hydrated electron reacts much faster than the formate radical with the chloramides, with rate constants close to those of diffusion-controlled reactions. The rate constants for the reaction of the hydrated electron can be compared to those measured for the small model molecule, NH_2Cl , where values of $2.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ were found [25,26]. Pulse radiolysis has also been used to measure hydrated electron reaction rates with chloramines and chloramides of amino acid related models, where rate constants in the range, $10^9 \text{ M}^{-1} \text{ s}^{-1}$ to $10^{10} \text{ M}^{-1} \text{ s}^{-1}$ were found [28].

Hence, the above studies have shown that both formate radicals and hydrated electrons react with HA and heparin chloramides to produce species with a broad absorption in the range 300-400 nm with maxima close to 320 or 330 nm and with associated extinction coefficients approaching $2300 \text{ M}^{-1} \text{ cm}^{-1}$. These spectral characteristics are very similar to those of free radicals produced from oxidation of the model compounds, N-methylformamide (NMF), dimethylformamide (DMF), N-methylacetamide (NMA) and N,N-dimethylacetamide (DMA) [45,46]. These molecules have an analogous structure to that in the glucosamine moiety of HA. When hydroxyl radicals were reacted with them, similar spectra with maxima between 340 nm and 380 nm and

extinction coefficients of $1000\text{--}2000\text{ M}^{-1}\text{ cm}^{-1}$ were observed [45]. These spectra were all attributed to free radicals having the structures $\text{RCONRCH}_2\cdot$ (where R can be H- or $\text{CH}_3\text{-}$). N-centred radicals , in contrast, did not absorb at all in this region , having maxima at $< 235\text{nm}$ [45]. Similar spectra were also observed for the oxidation of DMF by hydroxyl radicals ([45] and by sulphate anion radicals [46]. It seems clear therefore that the same assignments attributable to carbon-centred free radicals can be made in the current study on HAcI and HepCl, providing support for the 1,2 hydrogen shift mechanism proposed by Davies and co-workers [19] as shown in reaction 8 and in more detail in Scheme 2 in which a chloride ion is eliminated by one-electron reductants to form a nitrogen-centred radical in the first instance followed by rapid re-arrangement to form a C-2 radical on the glucosamine moiety. Both EPR data and ion-exchange chromatography to detect chloride in the one-electron reduction of glycosaminoglycan chloramides as well as in pulse radiolysis studies of the reaction of the hydrated electron with chloramines and amides confirm that chloride ion is produced in yields of 100% [47, 63].





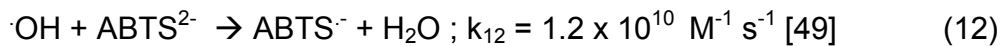
Scheme 2

Although reaction (8) must produce N-centred radicals in the first instance, these spectra indicate that carbon-centred radicals only are observed, consistent with a rapid internal rearrangement. The current study provides strong spectral evidence to support the formation of such carbon-centred radicals on the glucosamine moiety as shown in Scheme 2. As in the EPR study, the nitrogen-centred radicals must be short-lived as only carbon-centred radicals could be detected in the current study.

Oxidation by hydroxyl radicals

Kinetic data for the reaction of hydroxyl radicals with HACl and HepCl were obtained using the competing reactions:





In the pulse radiolysis of nitrous oxide saturated solutions containing $2 \times 10^{-5} \text{ M}$ ABTS^{2-} and HACl ($2.5 \times 10^{-4} \text{ M}$ to $1.0 \times 10^{-3} \text{ M}$), the changes in absorbance of the $\text{ABTS}^{\cdot-}$ radical at 415 nm were monitored up to 20 μs after the pulse. Oxygen as an impurity is reduced to very low levels by the use of nitrous oxide, allowing a 100% yield of hydroxyl radicals via reactions (1) and (2). Additionally, the $\text{ABTS}^{\cdot-}$ radical, used in the competition set out in reactions (9) –(11) does not react with oxygen. The measurement of rate constants for reactions (11) and (12) should not be affected by any low levels of oxygen. In these experiments, both the first order rate constant for $\text{ABTS}^{\cdot-}$ radical formation and the ratio of $\text{ABTS}^{\cdot-}$ radical absorbance at $[\text{HACl}] = 0 \text{ M}$ and at each $[\text{HACl}]$ were measured. Figure 7 shows the effect of HACl concentration on the ratio of absorbances, $\text{OD}(\text{ABTS}^{\cdot-})_0 / \text{OD}(\text{ABTS}^{\cdot-})_c$, at 415 nm at the end of the reaction of hydroxyl radicals with the competing HACl and ABTS^{2-} species, where $\text{OD}(\text{ABTS}^{\cdot-})_0$ and $\text{OD}(\text{ABTS}^{\cdot-})_c$ are the absorbances at $[\text{HACl}] = 0 \text{ M}$ and at other HACl concentrations respectively. Consideration of the competing reactions shows that:

$$\text{OD}(\text{ABTS}^{\cdot-})_0 / \text{OD}(\text{ABTS}^{\cdot-})_c = (k_{12}[\text{ABTS}] + k_{11}[\text{HACl}] + k_9[\text{HA}]) / k_{12}[\text{ABTS}]$$

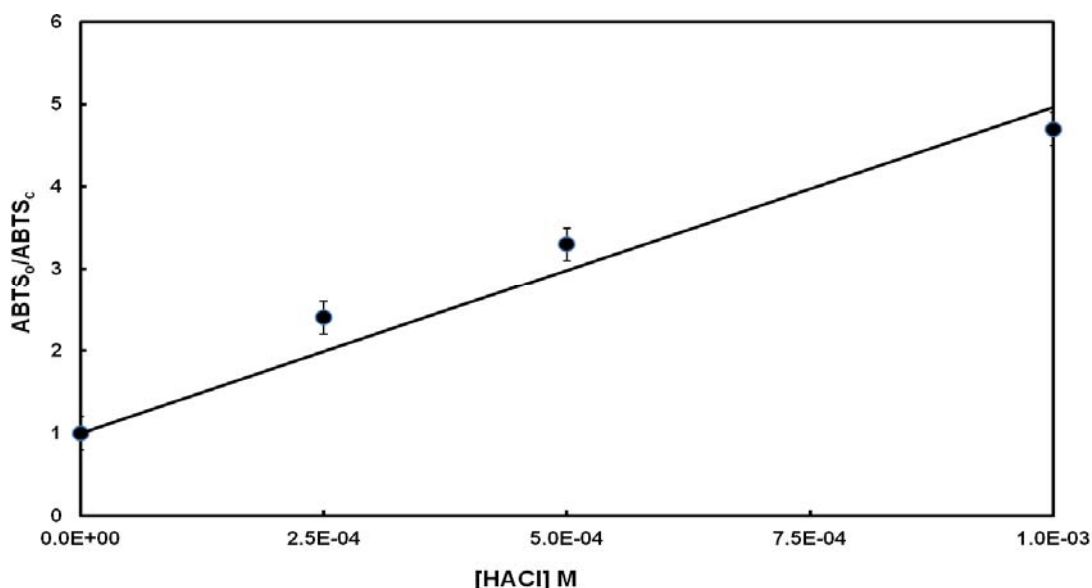


Figure 7 Effect of HAcI concentration on the absorbance ratio , $(OD(ABTS^{\cdot-})_0/OD(ABTS^{\cdot-})_c)$ (see text) at 415nm at the end of the reaction of hydroxyl radicals with 2×10^{-5} M $ABTS^{2-}$. (in nitrous oxide saturated solutions of 2×10^{-5} M $ABTS^{2-}$ containing 2.5×10^{-4} M to 1.0×10^{-3} M HAcI)

As a consequence of the method of preparation, the concentrations of HAcI and HA are in a fixed ratio where the ratio $[HA] / [HAcI]$ is 1.9.. Hence, the latter equation reduces to :

$$OD(ABTS^{\cdot-})_0/OD(ABTS^{\cdot-})_c = 1 + (k_{11} + 1.9 k_9) [HAcI] / k_{12} [ABTS]$$

From the slope of figure 7, and using the known k_9 and k_{12} values, a value of k_{11} of $(2.2 \pm 1.0) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ was calculated.

Similar experiments were also carried out for HepCl where the method of preparation produced solutions containing Hep and HepCl in a fixed ratio ($[Hep] = 0.25 [HepCl]$). The data (not shown) produced a values for $k (\cdot OH + HepCl)$ of $(1.6 \pm 0.3 \times 10^8) \text{ M}^{-1} \text{ s}^{-1}$.

Hydroxyl radicals react rapidly with both HA and heparin, with k values of $4.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ respectively. With the model monosaccharides, glucuronic acid and N-acetylglucosamine (sole constituents of HA), the rate constants are much faster at $1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [48]. Within the error limits, hydroxyl radicals have been shown to react with HAcI in the current study at a similar rate to HA itself, indicating that the N-Cl group is not a more specific target than N-H. Similarly, in the case of HepCl, the rate constant determined here ($1.6 \pm 0.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$), is sufficiently similar to the earlier value of $4.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ found for the parent, heparin, to indicate that the N-Cl group does not confer any selectivity of attack. Thus, it is likely that hydroxyl radicals will abstract hydrogen from the many C-H groups in these molecules as well as from the N-H groups. Most if not all these C-centred radicals, including any arising from re-arrangement of the N \cdot radical, will produce peroxy radicals. It has been shown previously that peroxy radicals of hyaluronan produced by reaction of hydroxyl radicals lead to about 15% less fragmentation [16].

Oxidation by carbonate radicals

Laser flash photolysis (excitation at 266 nm) of air-saturated 30 mM persulphate solutions containing 300 mM bicarbonate at pH 8.5 produced high yields of the carbonate anion radical, $\text{CO}_3^{\cdot-}$, via reaction (5) followed by reaction (13):



Reaction (13) is relatively slow, with rate constants of $2.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [50] and $9.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [32] having been reported. Hence, the high bicarbonate concentration ensures that this reaction is complete within approximately 5 μs or less. The carbonate anion radical, $\text{CO}_3^{\cdot-}$, exists in equilibrium with the protonated form, HCO_3^{\cdot} with a $\text{p}K_a$ of 7.9 [51].



Upon laser flash photolysis of the persulphate / bicarbonate solution , the carbonate anion radical was formed within several microseconds and its formation and decay were monitored kinetically at 600 nm where the extinction coefficient of the carbonate radicals is $1860 \text{ M}^{-1} \text{ cm}^{-1}$ [52]. Carbonate radical concentrations of 7.5 to 10 μM were produced in typical experiments.

Figure 8 shows the decay of $\text{CO}_3^{\cdot-}$ (at 600 nm) both in the absence and presence of the parent glycosaminoglycans , HA (2.5 mM) and heparin (5mM), the monosaccharide, N-acetylglucosamine (5 mM) and also the amino acid taurine (5 mM). (Only a single concentration of the glycosaminoglycans was used (as also for their chloramides, see below) since the sulphate anion radical would react at higher concentrations whilst lower concentrations would not compete with the self-reaction of the carbonate radicals). In the absence of substrates, the carbonate anion radical decays relatively slowly over the 12 ms timescale. It decays by self-reaction with a rate constant of $(1.0 \pm 0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ as determined from a plot of $1/[\text{CO}_3^{\cdot-}]$ v time (plot not shown). In the presence of taurine, the decay of $\text{CO}_3^{\cdot-}$ is much faster – a plot of $\log [\text{CO}_3^{\cdot-}]$ v time produced a rate constant for the reaction of $(1.0 \pm 0.1) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. This appears to be the first rate measurement for the reaction of carbonate radicals with taurine. The effects of HA, heparin and N-acetylglucosamine on the decay of the carbonate radical are less pronounced. Here, kinetic simulation was used to estimate the rate constants. In this relatively straightforward process,. Only two reactions were simulated, the self-reaction of carbonate radicals ($k = 1.0 \pm 0.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and the reaction of carbonate radicals with HA, Hep or NAcGlc. The values obtained were similar, ranging from $3.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ to $5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. Table 2 summarises all the kinetic data for these parent molecules.

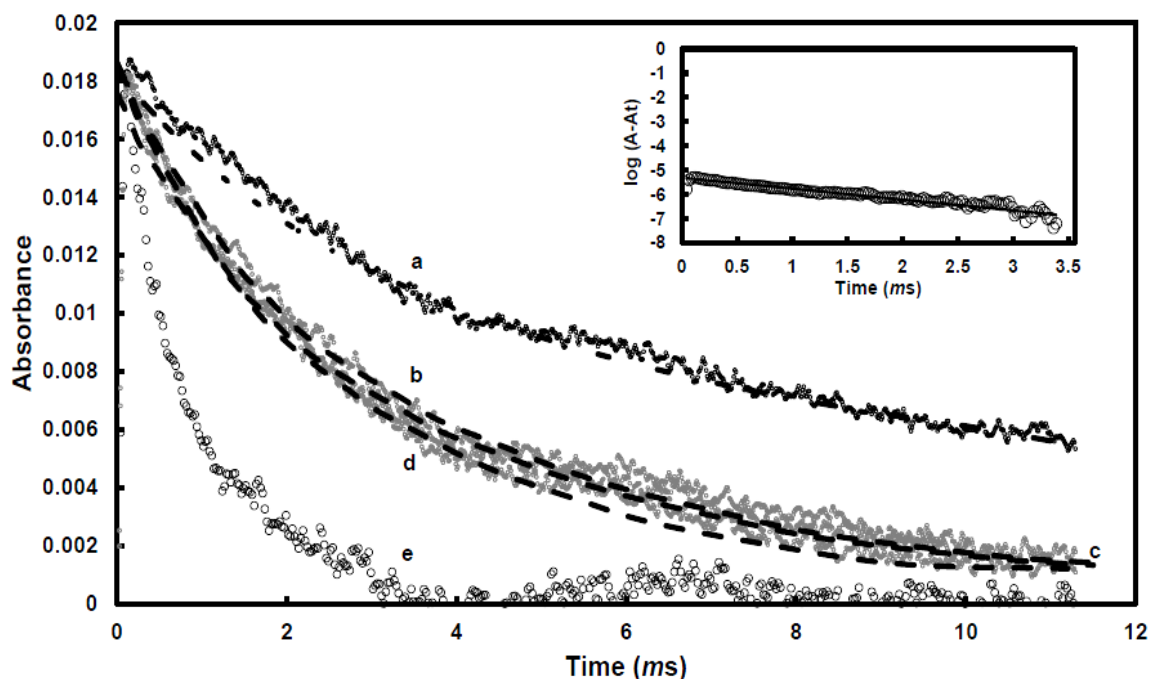


Figure 8 Decay of carbonate anion radical of absorbance at 600 nm in the presence of ; a) no substrate, b) 2.5 mM HA, c) 5 mM Hep, d) 5 mM NAcGlc, e) 5 mM Tau (the dotted lines show the simulated decays for a) , b) , c) and d)). (in air-saturated solutions of 30mM persulphate, and 300 mM bicarbonate at pH 8,5 , 0.01 M phosphate buffer). The inset shows the pseudo first –order plot for the faster taurine reaction.

“Substrate, S”	$k(\text{CO}_3^{\cdot -} + \text{S}) \text{ M}^{-1} \text{ s}^{-1}$
Hyaluronan	3.5×10^4
Heparin	5.0×10^4
Taurine	1.0×10^5
N- acetyl-glucosamine	5.0×10^4

Table 2 Rates of reaction of the carbonate radical with HA, Hep, NAcGlc and Tau.

Figure 9 shows the decay of $\text{CO}_3^{\cdot -}$ in the presence of the chloramides (including some proportion of the parent molecules) of HA, heparin and taurine. All decays are significantly faster than the decay of $\text{CO}_3^{\cdot -}$ in the absence of chloramide, with the fastest being observed in the case of TauCl. As before, kinetic simulations were carried out to allow for the self-reaction of carbonate radicals as well as with the chloramides and the parent biopolymers. The latter rates are known from Table 2 and hence, the rate constants for reaction with the chloramides can be

calculated in a relatively straightforward manner. Table 3 summarises the kinetic data for these chloramides which shows rate constants of $(1.2 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $(8.0 \pm 1.0) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for HAcI and HepCl respectively, values which are a factor of 2.9 and 1.6 x faster respectively than those determined for the parent molecules. Similarly, as for the parent amino acid, taurine, TauCl reacts significantly faster ($k = 1.4 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) than the glycosaminoglycan chloramides and also faster by a factor of 10 than the parent taurine.

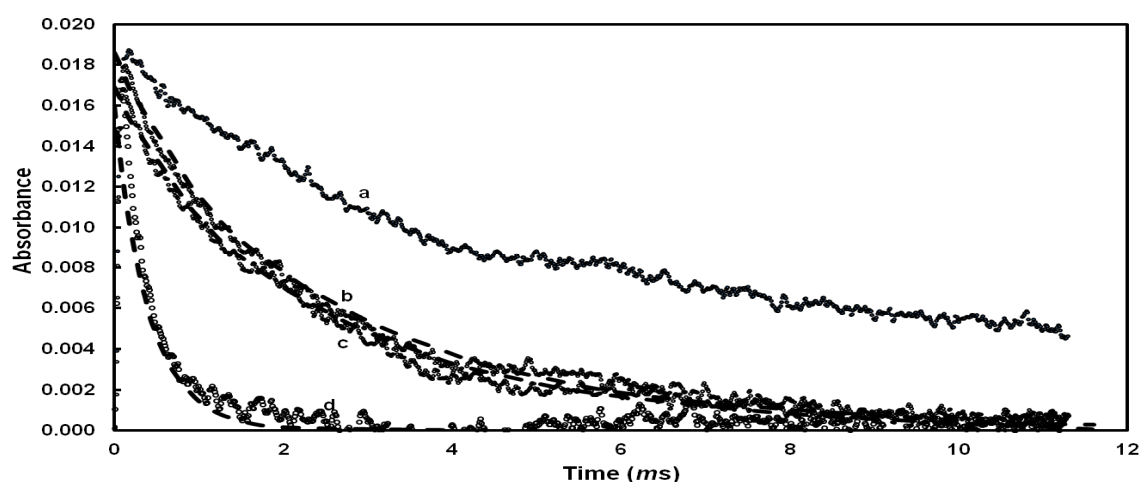


Figure. 9. Decay of carbonate anion radical of absorbance at 600 nm in the presence of (trace a) no substrate, (trace b) 3 mM HAcI/2 mM HA, (trace c) 3.8 mM HepCl/0.7 mM Hep, or (trace d) 3 mM TauCl (the dotted lines show the simulated decay for traces b and c), in air-saturated solutions of 30 mM persulfate and 300 mM bicarbonate at pH 8.5, 0.01 M phosphate buffer.

“Substrate, S”	$k (\text{CO}_3^{\cdot-} + \text{S}) \text{ M}^{-1} \text{ s}^{-1}$
HAcI	1.2×10^5
HepCl	8.0×10^4
TauCl	1.4×10^6

Table 3 Apparent rates of reaction of the carbonate radical with HAcI/HA, HepCl/Hep and TauCl (conditions as in Figure 10).

Hence, it is clear that the presence of the N-Cl group in both HA and heparin provides a significantly more attractive target than the N-H groups, with a factor

of 1.6 (HepCl) – 2.9(HAcI) times greater reactivity of carbonate anion radicals with HAcI and HepCl. Carbonate radicals are only moderately strong oxidizing agents and can be expected to be more selective than hydroxyl radicals. Hydroxyl radicals are strong oxidizing agents and are known to react rapidly with rate constants in excess of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ and do so by abstracting hydrogen at all the -CH(OH)- groups in simple carbohydrates, in oligosaccharides and in polysaccharides (reviewed in von Sonntag, 1986 [62]) . Their reactivity is thus largely unselective with regard to the site of attack. In hyaluronan, hydroxyl radicals are also highly reactive ($k = 8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [24]) and also likely to be unselective in their sites of attack with abstraction possible at 11 C-H bonds in HA . In contrast , the moderately strong oxidizing agent , the bromide radical ion, reacts at similar rates to the carbonate radical in the current study, and was found only to abstract hydrogen at C-1 of 2-deoxy-D-ribose , in a uniquely site-specific reaction [61]. By comparison, therefore, the presence of N-Cl appears , to present also a unique target in these glycosaminoglycan chloramides. Abstraction of the chlorine atom from N-Cl ,for example, would lead to an N-centred radical which would, at least in part, re-arrange rapidly to produce the C-2 radical on the glucosamine moiety, as in Scheme 2 or in part to produce C-4 radicals on the uronic acid moiety [19]. These radicals would react rapidly in the presence of oxygen to produce peroxy radicals. The various routes to fragmentation of glycosaminoglycan chloramides via peroxy radical intermediates have been discussed in detail by Rees et al, [19].

Conclusions

Localised excess production of HOCl has been implicated in a number of diseases that involve an acute or chronic inflammatory response (e.g. atherosclerosis, rheumatoid arthritis and asthma) [53,54] .There is evidence that

binding of myeloperoxidase to matrix components and cell-surface glycosaminoglycans directs oxidative damage towards these biopolymers[55,56]. At sites of inflammation within the ECM, activated phagocytes release the haem enzyme myeloperoxidase and produce high concentrations of the superoxide anion and its dismutation product, hydrogen peroxide via an oxidative burst. Myeloperoxidase is a highly basic protein and is known to bind, via electrostatic interactions, to negatively charged materials such as the polyanionic glycosaminoglycans [55]. In perlecan, for example, it binds to the heparin sulphate side-chains of this proteoglycan [57]. Myeloperoxidase reacts with hydrogen peroxide to form a mixture of hypochlorous acid and its anion hypochlorite. Relatively high concentrations of HOCl are likely to be formed *in vivo* under both physiological and pathological conditions. It has been estimated, for example, that activation of 5×10^6 neutrophil cells ml^{-1} generates 300–400 μM HOCl over 1–2 hours [58,59], with 2.5–5 mM HOCl produced at sites of inflammation [60]. In the ECM, where HA has a central structural role, it can be anticipated that HOCl will react preferentially with neighbouring proteins. There is little evidence for a direct reaction, in the ECM, between hypochlorous species and reactive N-H groups in HA and heavily-sulphated glycosaminoglycans such as heparan sulphate. However, such a theoretical model does not take into account accessibility of the reactive N-H groups in proteins and glycosaminoglycans in an extracellular environment or the possibility of localized chlorination and oxidation reactions when the cationic myeloperoxidase itself is localized preferentially at glycosaminoglycan moieties in

the extracellular matrix. The role of glycosaminoglycan chloramides in the potential fragmentation of these biopolymers by free radicals and reactive oxidative species remains open.

This study has demonstrated that the N-Cl group in glycosaminoglycan chloramides confers selectivity towards free radicals and reactive oxidative species. Reducing radicals are expected to be highly, perhaps 100%, selective in their attack, as shown in this study, as models of less strongly-reducing agents such as superoxide and glutathione disulphide anion radicals, by the exclusive reaction of formate radicals and hydrated electrons at N-Cl to eliminate chloride ions. The initially-formed nitrogen-centred radical re-arranges rapidly to form a carbon-centred radical, as shown in Scheme 2 (supporting the earlier proposal made in an EPR study [19]) probably also leading to a 100 % efficient fragmentation of the biopolymers. The potential *in vivo* oxidising radicals, in this study hydroxyl radicals and carbonate anion radicals, also show some significant degree of selectivity, particularly in the case of the biologically-relevant carbonate anion radical. This free radical is much more selective than hydroxyl radicals and is therefore much more likely to reach sensitive targets, such as those in the ECM. This study has shown that carbonate anion radicals are much more likely to react with N-Cl groups in the chloramides than with N-H groups, perhaps to react solely at the N-Cl group. Despite the large difference in charge density between HA and heparin, this study has not found any large effects of charge on reactivity, indicating that both non-sulphated and heavily-sulphated components

of the ECM are equally likely to be site-specific targets for reducing radicals and moderately strong oxidising agents such as the carbonate radical . The question remains open whether the greater degree of site-specific attack on ECM chloramides by both reducing agents and by oxidising agents such as carbonate radicals will lead to more efficient fragmentation than would be found for the parent biopolymers..

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